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Drosophila twin spot clones reveal cell division dynamics in regenerating imaginal discs

Anne Sustar [a], Marianne Bonvin [b], Margrit Schubiger [a], Gerold Schubiger [a,*]

[a] Dept of Biology, University of Washington, Seattle WA 98195, USA
[b] Swiss Science and Technology Council, Bern, Switzerland

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ABSTRACT

Cell proliferation is required for tissue regeneration, yet the dynamics of proliferation during regeneration are not well understood. Here we investigated the proliferation of eye and leg regeneration in fragments of *Drosophila* imaginal discs. Using twin spot clones, we followed the proliferation and fates of sister cells arising from the same mother cell in the regeneration blastema. We show that the mother cell gives rise to two sisters that participate equally in regeneration. However, when cells switch disc identity and transdetermine to another fate, they fail to turn off the cell cycle and continue dividing long after regeneration is complete. We further demonstrate that the regeneration blastema moves as a sweep of proliferation, in which cells are displaced. Our results suggest that regenerating cells stop dividing once the missing parts are formed, but if they undergo a switch in cell fate, the proliferation clock is reset.

Introduction

In *Drosophila* imaginal disc regeneration, like in salamander limbs, a blastema of dividing cells forms at the cut site and replaces lost patterns (Karpen and Schubiger, 1981). This type of regeneration is known as epimorphic regeneration. Understanding the nature of blastema founder cells may allow us to understand why regeneration is limited in mammals.

Upon limb amputation in salamanders, epithelial cells migrate over the wound and form a thickened structure called the apical epithelial cap (AEC). The AEC is thought to be functionally comparable to the apical ectodermal ridge (AER) of the normal developing limb, coordinating proximo-distal patterning and outgrowth with the expression of signaling proteins such as fibroblast growth factors (FGFs). Beneath the AEC, cells with an undifferentiated morphology accumulate, forming the blastema (reviewed by Tweedell, 2010).

Where do these blastema cells come from? Generally, it has been thought that cells at the salamander limb stump dedifferentiate to an embryonic-like, pluripotent state. However a recent lineage study challenged this idea. Kragl et al. (2009) transplanted groups of green fluorescent protein (GFP) expressing cells onto non-GFP-expressing hosts. The hosts' limbs were amputated, and the GFP-expressing cells were traced through regeneration. They observed that while many tissue types could contribute to regenerated structures, they did not observe any cases in which the presumed founder cells underwent major changes in identity. They concluded that the blastema is a heterogeneous population, where cells undergo limited dedifferentiation, remember their tissue origin, and only redifferentiate their own cell type. Still, the topic is controversial with additional suggestions that transdifferentiation is possible, or even that stems cells may be involved (reviewed by Mariani, 2010; Tweedell, 2010; Whited and Tabin, 2010). Active research efforts are aimed at settling these questions, for example, by performing single-cell clonal analysis to better describe the number of founder cells and their individual proliferative and developmental capabilities (J. Whited, pers. comm.).

The genetic tools available in *Drosophila* allow us to address many of these questions directly. *Drosophila* imaginal discs, the larval precursors for adult fly appendages, have been used as a model system to establish fundamental principles about development and patterning, including intercalary regeneration (Haynie and Bryant, 1976). In contrast to vertebrate model systems where regeneration depends on a dialog of signals between different germ layers, *Drosophila* imaginal discs have an advantage in their simplicity; the disc epithelium is mainly composed of a single ectodermal germ layer. Although the disc cells are not terminally differentiated, they are rigidly determined to form specific structures (Schubiger, 1971), and express differentiation genes, for example, for specialized proneural identities (reviewed by Treisman, 2004). Despite this rigid determination, when imaginal discs are fragmented and allowed to proliferate, they can regenerate and replace the missing structures. Thus the disc shows developmental plasticity. This plasticity is even more apparent in cases where regenerating disc cells take on the fate of a different imaginal disc, for example, switching from eye to wing, in a process termed transdetermination (Hadorn, 1978). From clonal analysis it was deduced that only three to five founder cells are responsible for the formation of the regenerated and

* Corresponding author.
E-mail address: gerold@uw.edu (G. Schubiger).

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the transdetermined structures (Gehring, 1967; Gibson and Schubiger, 1999).

What is the nature of these founder cells? Studies by Hadorn and co-workers in the 1960s favor the notion that some cells in the disc have a higher developmental plasticity. Disc fragments were injected into an adult host where they grew. Subsequently, large disc fragments were recovered, re-cut and re-transplanted. This process was repeated hundreds of times. Surprisingly, though most of the cell lines died out over this time, some lines from all different types of discs remained healthy, continued to divide, and maintained the competency to differentiate adult structures, even after 5 years. They concluded that some cells were immortal (Hadorn, 1978). Since immortality is one of the hallmarks of stem cells, this finding supports the idea that there may be stem cell-like cells in the disc epithelium that are activated during regeneration. Other observations, however, favor a change in the state of determination at the onset of regeneration (Schubiger and Alpert, 1975; Sustar and Schubiger, 2005; Schubiger et al., 2010), in a mechanism more comparable to what has been described in salamander limb regeneration.

With the advent and improvements of twin spot generating systems in *Drosophila*, where the progeny of two daughter cells can be monitored (Griffin et al., 2009), we were able to follow the proliferation of sister clones arising from the same mother cell in the blastema. We were particularly interested to see if the two clones differed in their cell number, indicating unequal properties of the two daughter cells. This study also allowed us to distinguish regenerating cells from those not participating in regeneration, and to follow regenerating versus transdetermining cell populations. We determined the number and doubling time of cells during regeneration and observed that transdetermined cells continue to divide after regeneration stops. This result, together with the observation that transdetermined cells can repeatedly switch fate, leads us to speculate about how imaginal disc cells attain immortality. We also observed that regenerating clones break up, indicating that regenerating cells become more mobile. We hope that principles uncovered in the imaginal disc model system may also apply more broadly, and may provide a template for future studies in other animals.

Materials and methods

Fly stocks

Wild-type flies were of the Sevelen strain. *y,w, hsflp*^{122};; *GrR* $^{\#18-2}$/ *RGr* $^{\#25-3}$ flies were used for generating RFP and GFP labeled twin spots (Griffin et al., 2009). MARCM clones were generated with the stock *hs-flp, UAS-CD8::GFP; +; FRT82B/Tub-Gal4, FRT82B Tub-Gal80* (L. Buttitta). For some experiments *ss*^{732}*-LacZ* (Emmons et al., 2007) were added to these stocks to allow us to identify distal antenna (basal cylinder and segment 3) and distal leg fates (tarsal segments 1–4). Alternatively, *vg (Boundary Enhancer* $^{(BE)}$*)-LacZ* (Williams et al., 1993), an enhancer that is required for leg to wing transdetermination (Maves and Schubiger, 1998), was used to identify presumptive wing margin, wing hinge and notum.

In vivo disc culture and induction of twin spots

Eggs were collected in 30–120 minute intervals following a 60 minute pre-collection to eliminate over-aged embryos. Animals were raised at 25 °C on standard fly medium. At early wandering stage, about 108 h after egg deposition (AED), discs were dissected in Ringer's solution from donor larvae, fragmented with tungsten needles, and injected into the abdomens of 1-day old adult female hosts (Schubiger, 1971). Twin spots were induced 1–2 h after fragmentation/transplantation by heat shocking the host flies for 20 min in a vial submerged in a 37 °C water bath. This protocol generated about 10–30 clones per disc, a density in which clones are separated clearly by non-clonal material. Males were added to the vials to fertilize the female hosts. Fragments were left in in vivo culture for 1, 2, 3, 4, 7 or 13 days.

We observed a low level of spontaneous clones, about 1 twin spot per 10 discs, in non-heat-shocked discs from freshly dissected larvae. In disc fragments that were transplanted for in vivo culture, however, nearly every disc contained a few small, spontaneous twin spots (90%, n = 20). Thus fragmentation and/or transplantation stress seems to induce a significant number of clones. We accommodated these spontaneous clones because, in our experiments, the heat shock was applied at approximately the same time as fragmentation. But the occurrence prevented us from varying our protocol to induce clones at earlier or later time points in the experiments.

Immunohistochemistry on twin spots

After in vivo culture, discs were fixed in 4% formaldehyde in PBS for 20 min in a deep depression slide. Discs were rinsed with PBS-Tx and blocked for 15 min in 5% normal goat serum (Sigma). To amplify the signal of the twin spots, we used mouse anti-GFP (1:500, Invitrogen) and rabbit anti-DsRed (1:500, Clontech) followed by Alexa Fluor goat secondary antibodies (1:500 each, Molecular Probes). To monitor disc patterning, we used rabbit anti-βgal (1:1000 Cappel), rabbit anti-Vg (S. Carroll, 1:100), mouse anti-Engrailed (DSHB, 1:50), mouse anti-Mmp1 (DSHB 1:100), or rat anti-Elav (DSHB, 1:30). When triple labeling required the use of two mouse or two rabbit primary antibodies, we reduced cross-hybridization by performing sequential staining: one set of primary and secondary staining was completed, followed by 2 h of thorough washes in PBS, and an immediate second round of primary and secondary staining. Finally, discs were incubated in DAPI (0.5 μg/ml in PBS) for 20 min, rinsed 3× in PBS, and mounted in Fluromount.

EdU

EdU was performed using the Click-iT EdU imaging kit (Invitrogen C 10338). In Fig. 3, EdU was incorporated mid-in vivo culture by etherizing the hosts and injecting them with a small amount of 1 mM EdU in Ringer's solution. In all other experiments, discs were dissected and incubated in 10 μM EdU in Ringer's for 30 min (N. Zielke, pers. comm.). Following EdU labeling, discs were fixed for 20 min in 4% formaldehyde, and antibody staining was performed according to standard protocol (see above). Finally, discs were washed twice in PBS-Tx, incubated in 1x Click-iT reaction cocktail for 30 min, washed thoroughly in PBS, stained in DAPI, and mounted.

BrdU

Discs were incubated for 20 min in 100 μl of 20 μg/ml of BrdU in PBS in a deep depression slide dish, washed with PBS and fixed in 4% formaldehyde for 20 min, hydrolyzed in 2 N HCl for 1 h, and rinsed in PBS-Tx before antibody staining with mouse anti-BrdU (1:200, Becton-Dickinson).

Twin spot data analysis

Each experiment was performed 1 to 3 times until we collected a sufficient number of clear cases in which a GFP/RFP twin spot was found in the regenerated/transdetermined region. For example, a clear case of a regenerated 3/4L leg fragment (Fig. 4) is defined as displaying two *ss* rings, one of which is associated with a high number of small clones (the non-regenerated part), the other with one or a few large twin spots (the regenerate). The twin spot generating system also produces yellow and unlabeled twin spots that are not usable for our analysis. Table 1 displays a representative case of the yield of one experimental series.

Table 1
Yield of selected cases of eye and leg regenerates from a typical experiment after 7 days of in vivo culture (Fig. 4B, B′, D, D′).

	Eye, n (%)	Leg, n (%)
Total analyzed implants	209 (100)	132 (100)
Number of regenerates, i.e. *ss* positive	146 (70)	116 (88)
Implants with sufficient number of clones for analysis (usually between 10 and 30)	129 (62)	30 (23)
Implants with a GFP/RFP twin spot overlapping the *ss* domain	4 discs (1.9)/5 clones	6 discs (4.5)/9 clones

Discs were imaged with BioRad Radiance 2000, Leica SP1 or Leica SP5 confocals. ImageJ-1.41 was used for image processing and to make 3D projections of the z-series. In each disc we counted a representative area of DAPI-labeled nuclei to estimate cell density. We used Image J's freehand selection tool to outline each clone and measure its area. We multiplied these area measurements to the cell-density to estimate cell number per clone. The $\log_2$ of cell number per clone allowed us to estimate the number of cell doublings. To estimate the cell doubling time, we used (duration of culture, in hours)/[$\log_2$ (cell number)]. Values were analyzed using a two-tailed Mann Whitney U-Test and assuming statistical significance at $p<0.05$.

All images presented are complete projected z-series, with the exception of Figs. 4B and 5A–B, in which partial z-series were used to emphasize the most relevant clonal material.

Results

Disc regeneration and transdetermination are achieved with a sweep of cell proliferation

Previously our lab focused on regeneration in the prothoracic leg imaginal disc and was able to identify genes that function in the formation and maintenance of the regeneration blastema (Abbott et al., 1981; McClure et al., 2008). Here, to complement our experiments with the leg disc, we took advantage of the even simpler morphology of the eye-antennal disc. The eye disc is flat and thus physically easy to cut, and the concentric folding of the regenerated antenna is morphologically distinct and identifiable from the eye disc (Fig. 1A, D). Also, the flat eye morphology produces cell clones that are more two-dimensional and straightforward to analyze.

In preliminary experiments, we asked whether eye-antennal regeneration, as in the leg disc and other model systems, is accomplished by a blastema. We monitored S-phase in disc cells with short pulses of either BrdU or EdU incorporation. Control eye-antennal discs were dissected from early-wandering larvae, approximately 108 h after egg deposition (AED) (Fig. 1A, D). In the antennal primordia and the anterior-most cells of the eye disc, BrdU and EdU were incorporated uniformly (100%, n = 10). More posteriorly, incorporation was patterned, corresponding to the sweep of the morphogenetic furrow across the eye disc and the progressive differentiation of ommatidial precursors (Thomas et al., 1994); BrdU and EdU were absent in the furrow itself, where cells arrest in G1, while BrdU incorporation was strong in a band of cells immediately posterior to the furrow. Finally, in the most posterior part of the eye disc, cells were labeled randomly over a wide field, in cells fated to become cone and pigment cells (Wolff and Ready, 1991).

We cut eye-antennal discs from early-wandering wild-type larvae and cultured the eye fragment in vivo for 1 day or 3 days. At the end of the culture period, we incubated the discs in BrdU or EdU to monitor cells in S-phase, and co-labeled them with Elav to visualize photoreceptor cells, allowing us to orient the disc (Fig. 1B, C). After 1 day of in vivo culture the overall proportion of cells in S-phase was reduced, except in a small medial pocket of cells anterior to Elav expressing cells, near the cut site (40%, n = 15; Fig. 1B, E, arrowheads).

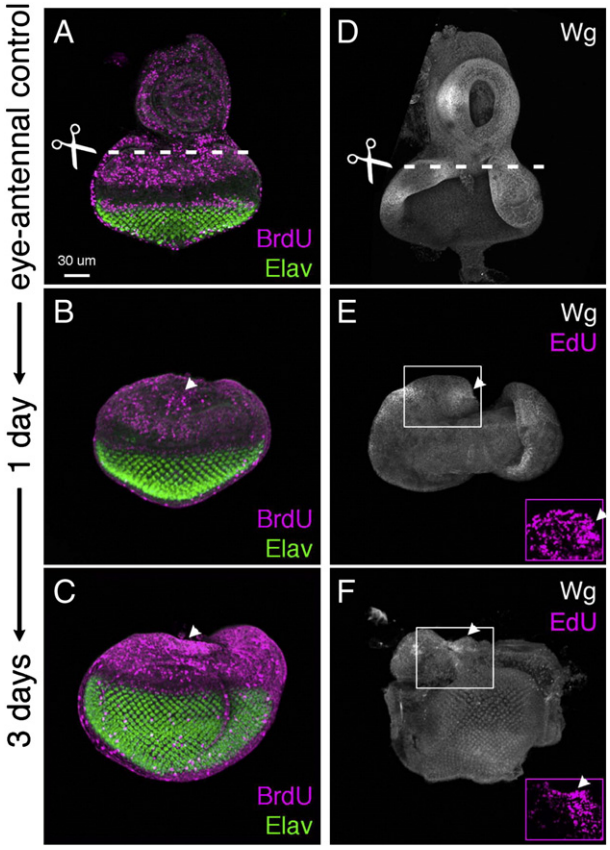

Fig. 1. The eye disc forms a regeneration blastema at the site of the cut where Wg is upregulated. Wild-type eye discs were cut (A, D) and cultured in vivo for 1 day (B, E) or 3 days (C, F). At the end of culture, BrdU (A–C; magenta) or EdU (E–F; magenta) labeled cells in S-phase. Elav (A–C; green) expression marks photoreceptor cells. During regeneration, the proportion of cells in S-phase is reduced except in medial cells, anterior to the photoreceptors (arrowheads; B,C), where Wg is upregulated (arrowheads; E, F). Anterior is up. Scale bar = 30 μm.

The labeling was stronger and encompassed a larger area after 3 days of culture (75%, n = 24; Fig. 1C, F). Thus, upon fragmentation of the eye disc, a cell population near the wound enters S-phase. This indicates that, like salamander limb regeneration, eye disc regeneration is accomplished by a regeneration blastema.

Also, in many regenerating eye discs a weaker but distinct band of BrdU incorporation at the morphogenetic furrow was maintained (Fig. 1B, C). The frequency of furrow labeling diminished after a longer culture period, from 63% at 1 day to 29% at 3 days. To test if the furrow continues to progress in in vivo culture, we counted the rows of differentiated ommatidia precursors, as visualized by Elav staining. At the time of the cut, eye discs had an average of 8 rows of ommatidia (±1; n = 16; Fig. 1A). After 1 day and 3 days of in vivo culture, discs had 13 rows (±3; n = 15; Fig. 1B) and 22 rows (±3; n = 24; Fig. 1C), respectively, indicating that the morphogenetic furrow continues to move anteriorly during in vivo culture. Thus, the morphogenetic furrow progresses normally, whether or not the antenna disc is present.

In many systems, including leg imaginal discs, canonical Wingless (Wg/Wnt) signaling is necessary and sufficient for regeneration (Johnston and Schubiger, 1996; Kawakami et al., 2006; Smith-Bolton et al., 2009; Stoick-Cooper et al., 2007). In fragmented leg discs Wg upregulation is observed within 24 h at the cut site where the blastema is forming, (Gibson and Schubiger, 1999; McClure et al., 2008). Therefore we asked whether Wg is also upregulated in regenerating eye discs at the site of the blastema. In control eye-antennal discs at the time of the cut Wg is expressed only at the lateral margins of the eye anlage and in a ventral-anterior wedge of the antennal disc (Fig. 1D;

Treisman and Rubin, 1995). One day after in vivo culture, Wg was upregulated near the cut site with high frequencies (77%, n = 22; Fig. 1E), and frequently overlapped with EdU. After 3 days of in vivo culture, ectopic Wg was still seen in most regenerates (66%, n = 15; Fig. 1F). Thus, Wg is rapidly induced at the wound site and was expressed in the blastema for at least 3 days.

What structures does the fragmented eye regenerate? When an eye disc is separated from the antennal disc and cultured in vivo, regeneration of antennal structures, as indicated by differentiated cuticle, occurs in 43% of cases (Gehring and Schubiger, 1975) and transdetermination to wing occurs in 27% of cases (Schmid, 1985). Surprisingly, cuticle analysis indicated that during antennal regeneration, more distal segments like arista are specified before the more proximal segments are complete (G. Schubiger, data not shown/unpublished observations). Other than this, little is known about the sequence of pattern regeneration in fragmented eye discs. To address this gap, we fragmented eye discs and followed the re-patterning using molecular markers after different durations of in vivo culture.

To monitor the regeneration of antennal precursors we stained discs for *spineless*^{732}*-LacZ (ss-LacZ*, Emmons et al., 2007), a reporter for distal antennal fates, and to monitor transdetermination, we followed the expression of Vestigial (Vg), a wing selector gene (Williams et al., 1991). Neither of these genes is expressed in unfragmented eye discs (Fig. 2A, n = 10). Eye disc fragments were cultured in vivo for 2 days, 4 days, or 7 days. Upon recovery, we also monitored the blastema with a 30 minute pulse of EdU. *ss-LacZ* expression was first seen at 4 days with a 33% frequency, increasing to 77% after 7 days of in vivo culture. During this time, the median area of *ss-LacZ* expression increased 2-fold. Vg expression was observed with a frequency of 39% after 2 days of in vivo culture, increasing to 46% after 4 days and 87% after 7 days (n = 31, 24 and 31, respectively, Fig. 2B–D). Between 4 days and 7 days of in vivo culture, the median area of Vg increased fivefold.

At the two earlier time points – 2 days or 4 days – if Vg or *ss-LacZ* expression was present the blastema overlapped in these regions in 96% of cases (Fig. 2B–C). But strikingly, after 7 days, proliferation in the *ss-LacZ* region was only seen in 29% of the cases, while proliferation in the Vg region was seen in 81% of cases; Fig. 2D). Our results suggest that as the disc re-patterns, transdetermining cells continue to proliferate after regeneration stops.

A fundamental question in regeneration is whether groups of cells proliferate and become re-specified as a group, or whether a sweep of cell division induces specification one identity at a time. According to the first hypothesis, the cells in the blastema become determined into zones with specific proximo-distal identity early on, as in salamander limb regeneration. Each zone proliferates to expand the blastema before the cells differentiate (Echeverri and Tanaka, 2005). In the second hypothesis, cells proliferate within a limited zone, then exit into a non-proliferative zone to undergo differentiation, as in zebrafish fin

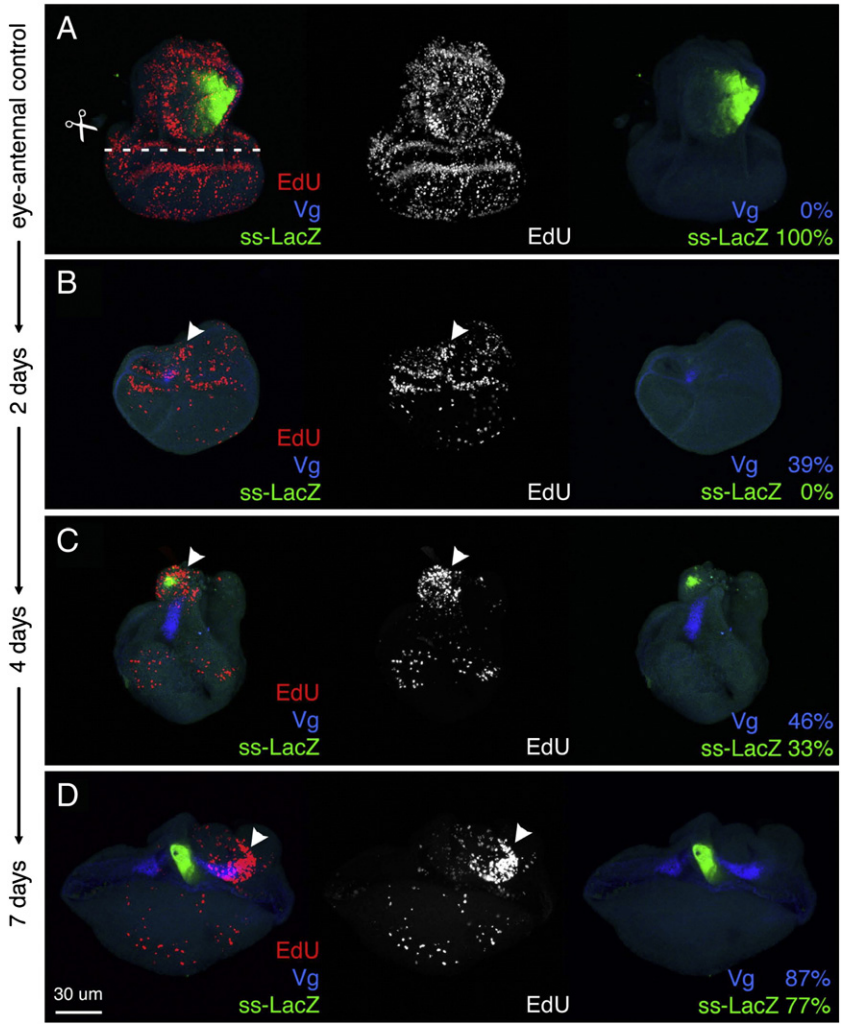

Fig. 2. The blastema is formed at sites of newly regenerated and transdetermined structures. EdU (red), Vg (blue) and *ss-LacZ* (green) in control eye-antennal discs (A), and in eye disc fragments after 2 days (B), 4 days (C) or 7 days (D) of in vivo culture. Anterior is up. Arrowheads (B–D) indicate sites where concentrated EdU incorporation is observed adjacent to the cut site and overlapping *ss-LacZ* and/or Vg expression. Scale bar = 30 μm.

regeneration (Poleo et al., 2001). While our results suggest a sweep of cell division, i.e. that the blastema shifts from regenerating cells to transdetermining cells, we investigated this further by taking advantage of EdU and BrdU to label S-phase cells at different time points. If the first hypothesis was true, we would expect to see BrdU and EdU labeling intermingled, indicating that the blastema was expanding equally. If the second hypothesis was true, we would expect to see EdU and BrdU label in separate zones.

In a set of preliminary experiments we transplanted discs and, 6 h later injected EdU into the hemolymph of hosts. After one or two days of in vivo culture, we recovered the discs and observed robust EdU labeling in every case (n = 7 and 16, respectively). We also tested the duration of labeling by injecting EdU into the hosts and, 6 h later, transplanting discs. The discs were cultured for 2.5 h. Of 16 discs, 14 showed no or only faint EdU label and only 2 had clear label, while all of the hosts' ovarioles had blazing EdU label. Based on these results, we believe that an injection of EdU into the host is metabolized in about 6 h, and thus provides a relatively short pulse of EdU.

We then fragmented eye discs and labeled the blastema at 3 days by injecting EdU into the host flies. After 7 days of culture, we recovered the discs and bathed them in BrdU for 30 min. In these discs (n = 4, Fig. 3A), we observed EdU and BrdU labeling in two highly concentrated neighboring regions that did not overlap with each other. In a similar experiment we injected EdU at the onset of regeneration and gave a pulse of BrdU 4 days later, at the end of the culture period. 12 of the 15 discs had non-overlapping patches of EdU and BrdU label. One interpretation is that EdU label was diluted in the most prolific cells, and that the two populations came from the same lineage. We interpret our results as favoring the second hypothesis, that re-specification of new fates is achieved in a sweep of proliferation.

Results from leg disc fragments are consistent with this idea. We induced GFP-labeled clones in 3/4 lateral (3/4L) fragments at the time of the cut and gave an EdU pulse after 7 days in vivo. In 89% leg discs (n = 9; Fig. 3B) we observed that about half or less of the cells in large clones (i.e., in the regenerate) were labeled with EdU, indicating that most of the cells in the clone were no longer participating in regeneration. Considering this along with the evidence above, the most parsimonious interpretation is that regeneration is achieved as a sweep of cell proliferation.

Regenerating sister clones are symmetric

Our experiments with the eye fragment indicate that regeneration occurs in a sequential manner, as a sweep of proliferation. Previously, clonal analysis in the regenerated cells of the 3/4L leg disc fragments showed that a small number of founder cells proliferate to re-pattern the missing structures near the wound before cells of a more distant fate are regenerated (Abbott et al., 1981; Gehring, 1967). In imaginal discs cell proliferation is necessary for regeneration, as it is in all known systems except hydra (Cummings and Bode, 1984). What is not known, from any system, is whether both daughter cells participate equally in regeneration, with equal plasticity and proliferative potential. Therefore we used the twin spot generating system (Griffin et al., 2009) to follow the proliferation of sister clones arising from the same mother cell in the blastema. We were particularly interested to see if the two clones differed in their cell number, indicating unequal properties of the two daughter cells.

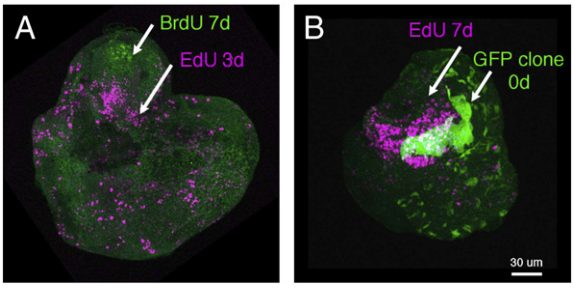

Fig. 3. Sweep of proliferation during regeneration. (A) Eye disc fragment showing EdU-labeled blastema cells after 3 days (magenta) and BrdU-labeled blastema cells after 7 days (green) in vivo culture. (B) A leg disc 3/4L fragment in which a GFP-labeled cell clone was induced at the time of the cut (green), and the blastema was labeled with EdU after 7 days (magenta) in vivo culture.

As a control, we examined twin spots in uncut eye-antennal and leg discs from early-wandering larvae that were subjected to in vivo culture. One to 2 h after transplantation, host animals were heat shocked to induce about 10 to 30 GFP and RFP-labeled twin spots per disc. After 7 days, we recovered and fixed the discs. DAPI staining allowed us to visualize nuclei and estimate the cell number in clones (see Materials and Methods). In eye discs, twins anterior to the furrow contained a median of 3 cells (n = 14 twins in 10 discs; Fig. 7A). In leg discs, twins contained a median of 4 cells (n = 19 twins in 10 discs; Fig. 7B). This indicates that discs from wandering larvae, if not stimulated by cutting, undergo about 1–2 divisions during in vivo culture. As a comparison, only about 1 extra division would be expected to complete disc growth in normal development (Fain and Stevens, 1982). We presume that the additional low level of division reflects maintenance of cells in the face of random cell death during in vivo culture (Bosch et al., 2008).

With that in mind, we used the same protocol in regenerating eye and prothoracic leg 3/4L discs. As in the above experiment, 1 to 2 h after cutting and transplantation, host animals were heat shocked to induce clones. After 1.5 days, 3 days or 7 days of in vivo culture, we recovered the discs. At the 1.5-day time point, discs were co-stained for Matrix metalloproteinase 1 (Mmp1) to identify clones in the early regenerate (Fig. 4A, C). As in salamander limb and zebrafish fin regeneration, Mmps are strongly upregulated at disc wound and can be used to monitor the early blastema (Bai et al., 2005; Yang et al., 1999). In *Drosophila*, Mmp1 is not expressed in any columnar cells of imaginal discs and is specifically upregulated at the wound within 4 h of fragmentation (Page-McCaw et al., 2003; McClure et al., 2008). Cell lineage tracing in fragmented discs has shown that the cells of the regeneration blastema arise from the wound edge (Bosch et al., 2008). At later time points, 3 and 7-days after in vivo culture, discs were co-stained with *ss-LacZ* to identify clones that overlapped with regenerated antenna or duplicated leg precursors (Fig. 4B, and D; Fig. 7A, B).

Each experimental series was performed one to three times, culturing between 100 and 300 disc fragments, until we scored a sufficient number of cases that met the criterion of having a distinct twin spot that co-localized with the regeneration marker (see Table 1).

In eye disc fragments, after 1.5 days of in vivo culture, the non-regenerating control twins, scored as twins anterior to the furrow but non-Mmp1-expressing, contained a median of 9 cells each (Fig. 4A, A′). In the same discs, however, the twins that partially overlapped with Mmp1 expression were much larger, with a median of 26 cells each (Fig. 4A). After 7 days of in vivo culture, non-regenerating twins contained a median of 13 cells each (Fig. 4B, B′). In the regenerating discs, twins that overlapped with *ss-LacZ* expression contained a median of 173 cells each (Fig. 4B, B′). This represents about 7–8 cell doublings in the regenerate, compared to only 2–4 cell doublings in the non-regenerate, similar to uncut disc controls.

We wondered if these results were specific to eye-antennal regeneration, or whether they could be generalized to other types of imaginal discs. Therefore we performed parallel experiments with the 3/4L prothoracic leg fragment. Here, the cell numbers in twin spots in the regenerate and non-regenerate were not significantly different from those in the eye disc (Fig. 4C, D; compare C′ with A′, and D′ with B′). One large twin spot had 496 cells after only 1.5 days and was clearly an outlier (Fig. 4C', 7B). Considering typical disc cell-doubling times, we believe it was a spontaneous clone that was induced earlier in development (see Materials and Methods). We conclude that in both eye-antennal and leg disc regeneration, clones in the regenerate undergo marked proliferation that is statistically different from the non-regenerating clones. The similarity in regeneration capacities of both discs is noteworthy since the

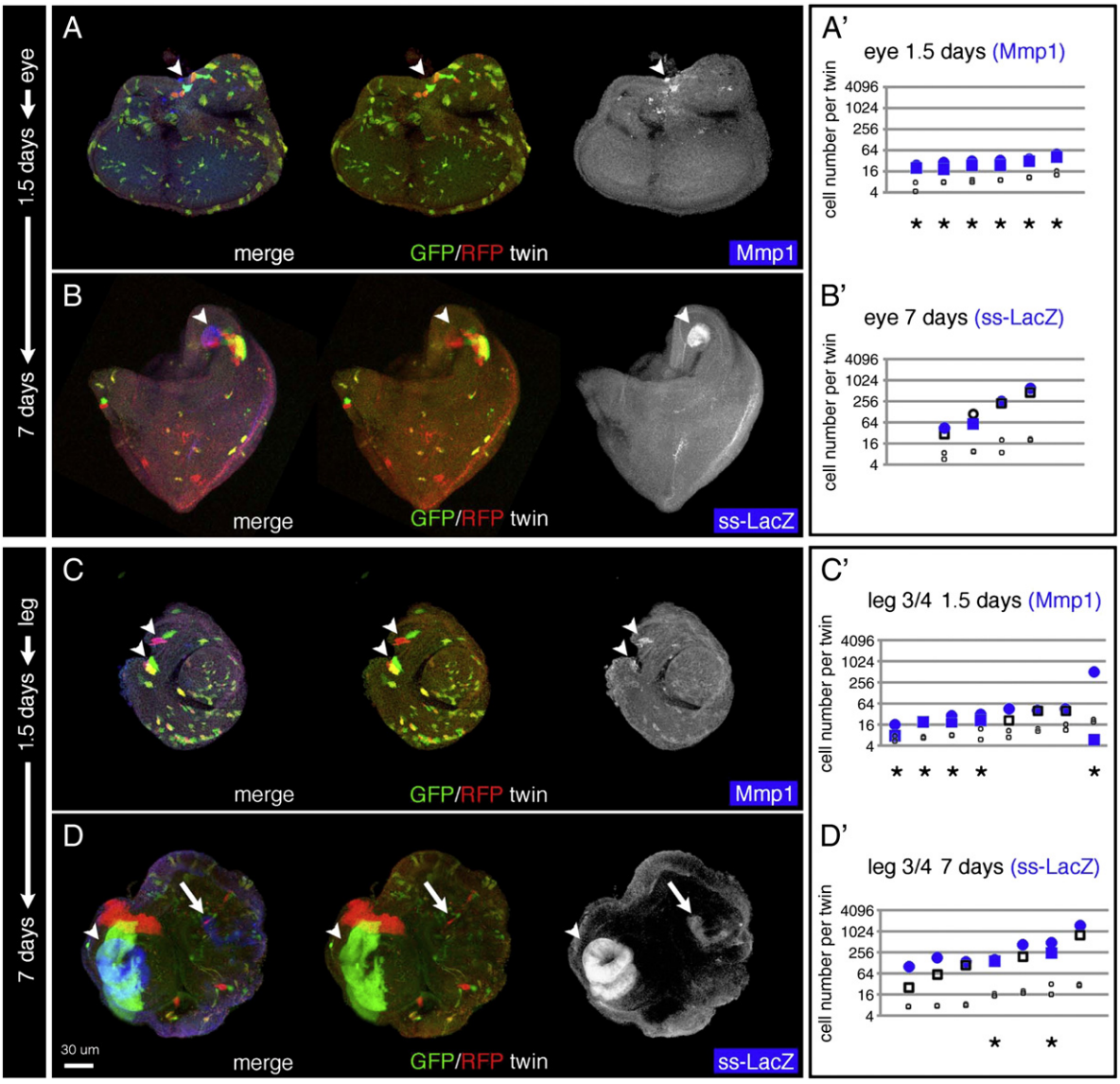

Fig. 4. Sister clones are symmetric in the regenerate. (A–D) RFP/GFP twin spots (red/green) in regenerated eye disc fragments (A–B) or regenerated leg disc 3/4L fragments (C–D) after 1.5 days (A, C) or 7 days (B, D) of in vivo culture. Mmp1 (A, C; blue) or *ss-LacZ* (B, D; blue) is expressed in the regenerate. Arrowheads (A–D) indicate a twin spot that overlaps with Mmp1 or *ss-LacZ* expression. (D) In leg disc fragments, two rings of *ss-LacZ* were observed, reflecting duplication of distal fates. One ring consistently showed strong expression and large clones, presumably representing the new part. *ss-LacZ* expression was weaker on the side that was associated with the nerve and the small clones and thus identified the non-regenerated region (D, arrow). As a control, we cultured uncut leg imaginal discs for the same time period and observed that *ss-LacZ* expression was weakened (data not shown). Scale bar = 30 μm. (A′–D′) Each graph represents data from selected cases (see Table 1) corresponding to the representative images in A–D. On the y-axis is the $\log_2$ cell number per twin clone. On the x-axis, pairs of twin clones are ordered from left to right by their sum's cell number. The larger symbols represent pairs from regenerating clones, the smaller symbols those from non-regenerating (control) clones. Blue indicates a clone that overlaps with Mmp1 (A′, C′) or *ss-LacZ* (B′, D′); a black symbol indicates a sister clone that does not. Cases where both sister clones overlap with the regeneration marker are highlighted with an asterisk.

starting material is quite different, especially with regard to the patterned proliferation that occurs in eye discs and not in leg discs. From here on, we concentrate on the twin spots in the regenerate.

In order to understand the dynamics of cell proliferation during regeneration, we analyzed a time point between 1.5 and 7 days. After 3 days of in vivo culture, twin spots in regenerating eye fragments had a median size of 111 cells, with a range of 5–7 doublings (n = 6). Twins in regenerating leg discs had a median size of 160 cells, with a range of 5–9 doublings (n = 6 each). These twins were slightly smaller, but not significantly smaller, than the twins that were analyzed after 7 days of in vivo culture. This suggests that most of the regenerative proliferation is completed within the first 3 days of in vivo culture (Fig. 7A,B).

Previous research in our lab found that regeneration in leg discs is accomplished with an estimated doubling time of 8 h, consistent with doubling times during normal third instar larval disc development (Gibson and Schubiger, 1999). In these experiments, clones were analyzed only at one time point, namely 4 days after clone induction. We asked whether shorter time points would give us more information about the dynamics of regenerative proliferation. We estimated cell-doubling times early during regeneration, at 18 h and 36 h after the cut, that is, the midpoints between the time of clonal induction (0 h) and clonal analysis (1.5 days and 3 days). Eye disc regeneration began with an 8 hour doubling time, slowing to 11 h. Similarly, leg disc regeneration began with an 8 hour doubling time, slowing to 10 h. These doubling times are comparable to the doubling times of imaginal discs reported during the early third instar (72 h AED to 84 h AED; McClure and Schubiger, 2005; Fain and Stevens, 1982) of normal development, when discs gradually slow from a 7-hour to a 10-hour doubling time. Later, during the late third instar, cells continue to slow to a 14 hour doubling time before arresting. The eye disc begins to slow at 84 h AED, whereas the leg disc does so after 96 h AED. We note that in regenerating eye discs, the doubling time was slightly, but significantly, longer than in the

regenerating leg disc. We propose that the difference seen between eye and leg disc fragments may correspond to the difference that is seen during the normal developmental program.

We were particularly interested to see if sister clones differed in their relative cell number, indicating unequal properties of the two daughter cells. We expressed each clone as the ratio of the cell numbers between sister clones, where a ratio of 1 indicates a symmetrically sized twin spot. After 1.5 days and 7 days of in vivo culture, twins in the eye-antennal regenerate had ratios of 1.2 and 1.4 (Fig. 7A). In 3/4L leg regenerates, the corresponding ratios were 1.5 and 2.0 (Fig. 7B). These ratios were not significantly different from normal levels of asymmetry observed in the non-regenerating (control) region of the discs. We conclude that when a cell divides to give rise to the regenerate, the daughter cells contain similar numbers of cells. This indicates that founder cells contribute equally to the regenerate.

While our results indicate that sister clones grow rather equally, we found that among the twin spots analyzed in eye-antennal regenerates, only one daughter per twin spot overlapped with the *ss-LacZ* expression in the newly regenerated antenna. Usually, this sister clone was slightly larger than its sibling (75% of cases after 7 days, n = 4; Fig. 4B′). In leg disc regenerates, we observed that when both daughter cells contributed to the *ss-LacZ* ring (29% of cases; n = 7 after 7 days, Fig. 4D′), the pairs tended to be more symmetric in terms of cell number, although the difference was not statistically significant. In the remaining leg disc regenerates, where only one sister clone overlapped with *ss-LacZ* expression, it was always larger than its sibling (Fig. 4D′). We conclude that while clones proliferate symmetrically during both eye-antennal and leg disc regeneration, morphogenetic information may also influence clone cell number.

Clone morphology suggests that regenerating cells are displaced

In normal disc development, dividing cells tend to maintain contact with their neighbors, leading to contiguous clones with a compact morphology (Gibson et al., 2006; Resino et al., 2002). In our experiments, while some twin spots in the regenerate were compact and had no obvious orientation, we observed many long, slender clones of only one or two cell diameters in width. For example, in leg 3/4L fragments that were cultured in vivo for 3 days and labeled with *ss-LacZ*, we observed long slender clones in 4 of 6 cases, and they were always oriented towards the center of the *ss-LacZ* ring. We interpret that the clone elongated towards the new structure along the proximo-distal axis (Fig. 5A). We made a similar observation in eye disc fragments that were cultured in vivo for 3 days and labeled with Elav to mark photoreceptor cells. Here, we observed long slender clones in 11 of 15 cases, and in most of these cases the clone was oriented perpendicular to photoreceptor rows and thus perpendicular to the former cut site (Fig. 5B). We hypothesize that two mechanisms could lead to elongated clone shape: oriented mitoses, or cell rearrangement and cell displacement. While oriented mitoses have been observed during wing disc development (Baena-López et al., 2005), and we cannot exclude this hypothesis, we asked whether there was any indication of cell displacement.

We previously reported cell displacement in normal wing discs when, during disc growth, cells from the peripodial epithelium are displaced to the underlying margin cells of the disc proper (McClure and Schubiger, 2005). Several years later, we reported a small but surprising level of clone separation specifically in this same region of wing and leg discs (Griffin et al., 2009). Therefore, we speculated that the forces involved in cell displacement may lead to clone separation, and we measured the frequency of clone separation in regenerating disc fragments. After 3 days of in vivo culture 17% of eye (n = 6, Fig. 5B) and 50% of leg discs (n = 6) showed clone separation, levels that are never observed during normal development. Here, clones were typically separated by 1 to 3 cell diameters. After 7 days, 20% and 80% (n = 10) of cases had clone separation, with many clones split into three or more smaller groups of cells, sometimes 10 cell diameters apart. Interestingly, clone separation was usually correlated with a long and slender clone shape (Fig. 5B). This indicates that cell displacement might contribute to the elongated shape. Also supportive of cell displacement are cases in which a large clone, found in the regenerate, was more than 20 cell diameters away from the presumed cut site (Fig. 5C).

We cannot exclude alternate explanations for the high frequency of clone separation. For example, wound healing is ongoing at the time of clone induction. Thus, the earliest events of clone separation could indicate that cell junctions are loosened while connections are made with new neighbors. Second, while we cannot completely exclude the possibility of late, spontaneous clone induction, we were conservative in our estimates and scored definite cases of clone separation only when split clusters of cells had the same clonal marker and no obvious twin spot neighbor. Finally, clone separation could be the result of cell death. While we have not investigated it here, we previously reported that blocking apoptosis does not change the doubling time of clones during *wg*-induced regeneration (Sustar and Schubiger, 2005). These results lead us to speculate that during regeneration, disc cells change adhesiveness and become more mobile than they are during normal development.

Sister clones that transdetermine are asymmetric

Abbott et al. (1981) previously analyzed clones in adult structures formed from regenerating leg fragments and demonstrated that clones could encompass regenerated leg structures as well as transdetermined wing elements. Here we wanted to validate our molecular markers, and to confirm Abbott et al.'s results that cells expressing *ss-LacZ*, our regeneration marker, are clonally related to cells that express our transdetermination marker, Vg. To address this issue, we fragmented discs and induced MARCM clones (Lee and Luo, 1999). After 7 days of in vivo culture, we stained the discs for *ss-LacZ* and Vg and looked for large clones that included both areas. In eye disc fragments, we identified 15

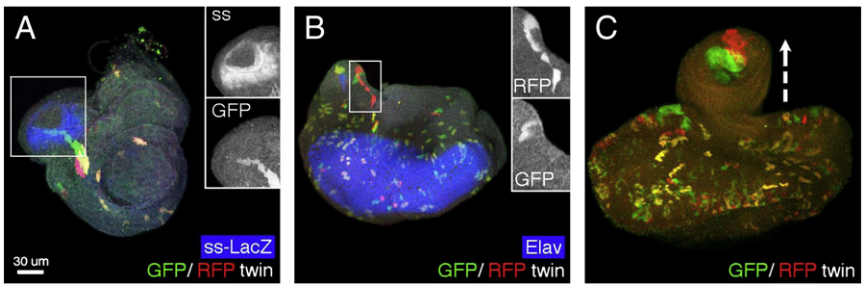

Fig. 5. Elongated and separated clones suggest that cells are displaced during regeneration. Regenerated 3/4L leg disc (A) and eye disc fragments (B, C) after 3 days (A, B) and 7 days (C) of in vivo culture. GFP/RFP twin spot clones (green/red) were induced at the time of the cut. (A) An elongated green clone in the regenerate is oriented towards the center of the *ss-LacZ* ring. (B) An elongated red clone at the cut site is perpendicular to rows of retina (Elav, blue), and is discontiguous compared to its green twin. (C) The dashed arrow indicates a large twin spot in the regenerate that is observed far from the presumed cut site (bottom of the dashed arrow). Scale bar = 30 μm.

cases in which a large clone overlapped with either *ss-LacZ* or Vg. Of these, 4 cases had a large clone that encompassed both markers (Fig. 6A). Likewise, in the leg 3/4L piece, we identified 15 cases in which a large clone overlapped with either marker, and only 1 clone encompassed both (Fig. 6B). Our results suggest that cells that make distal antenna or leg patterns *can* be clonally related to cells that transdetermine to wing, but that in the majority of cases they are not directly related. Thus, *ss-LacZ* expression is not necessary to activate Vg expression, and vice versa. It is important to consider that these two reporters only reflect a subset of leg, antenna and wing fates, and thus, our results probably underestimate the frequency with which regenerated and transdetermined structures are clonally related. Additionally, we cannot exclude the possibility that the Vg and ss-LacZ reporters are expressed transiently. Nevertheless, the variation in our results is supported by observations that regeneration and transdetermination are polyclonal events. Indeed, in both eye-antennal and leg fragments that were cultured in vivo for 7 days, we observed that the field of ss-LacZ and Vg-expression cells was never comprised of a single cell clone. Rather, the large clones occupied roughly 20%–50% of the ss-LacZ or Vg-expressing area. This is consistent with previous estimates that 3–5 founder cells give rise to the blastema (Gehring, 1967; Gibson and Schubiger, 1999).

While our results in Fig. 4 indicate that regenerating twins proliferate more or less symmetrically, we wanted to know if this was also the case when one of the sisters had transdetermined. We examined twin spot clones in transdetermining cells of eye (Fig. 6C) and leg 3/4L fragments (Fig. 6D) after 7 days of in vivo culture, that were labeled with Vg or *vg-LacZ* to identify clones that transdetermined to wing. Clones that overlapped with Vg contained a median of 1330 cells in the eye fragments (n = 6; Fig. 6C′) and 769 in the leg fragments (n = 4, Fig. 6D′). Not only were they larger than the control clones, but they were also significantly larger than their sister clones that did not overlap with Vg. In one case with an eye fragment, both sisters of a clone overlapped with Vg, and this pair of sister clones was symmetric in cell number (Fig. 6C′).

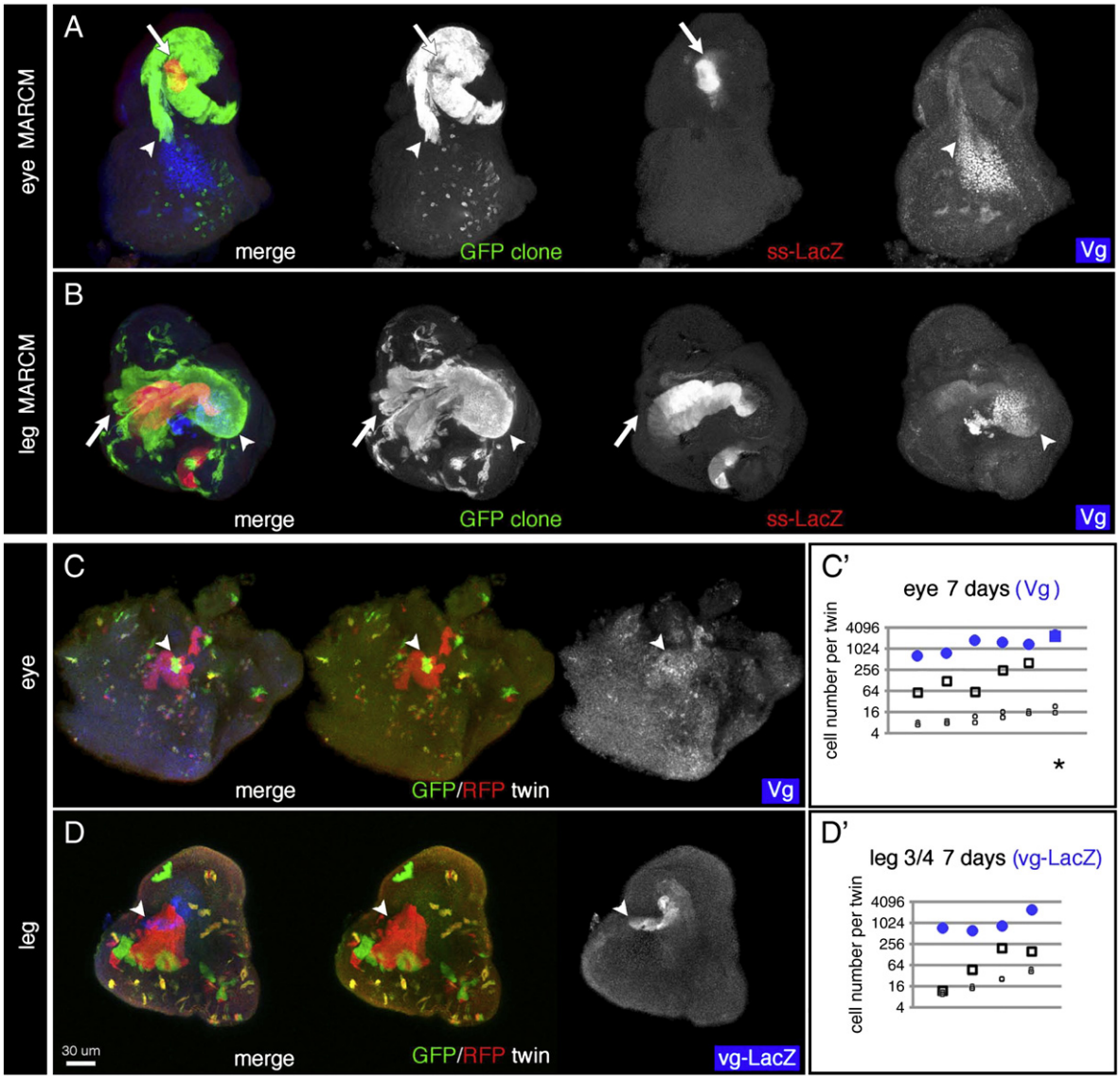

Fig. 6. Transdetermining sister clones are asymmetric and can be clonally related to regenerating cells. Regenerated eye disc fragments (A, C) or regenerated leg disc 3/4L fragments (B, D) after 7 days of in vivo culture. MARCM clones (green; A–B) or RFP/GFP twin spot clones (red/green; C–D) were induced at the time of the cut. Vg or *vg-LacZ* (A–D; blue) is expressed in cells that transdetermine to wing. *ss-LacZ* is expressed in cells that regenerate missing antenna (A) or distal leg (B). Arrowheads (A–D) indicate a clone that overlaps with Vg or *vg-LacZ*. Arrows in A and B indicate a clone that overlaps with *ss-LacZ*. Scale bar = 30 μm. Graphs (C′, D′) represent data corresponding to the representative images in C and D. The larger symbols represent pairs from transdetermining clones, the smaller symbols those from non-transdetermining (control) clones. Blue indicates a clone that overlaps with Vg or *vg-LacZ*, a black symbol indicates a sister clone that does not. A case where both sister clones overlap with the transdetermination marker is highlighted with an asterisk.

Looking at these cases in the context of the experiments presented in Fig. 4, we observed that transdetermined twins grew larger than in any other type of experiment, and also were more asymmetric (Fig. 7A). We noted this same trend in the leg (Fig. 7B) and studied additional time points to address the following questions: do transdetermined clones proliferate asymmetrically early, or only later? Does the asymmetry become more severe if we prolong the culture period? We cultured 3/4L fragments in vivo for 3 days and stained them for *vg-LacZ*, and observed that twin spots which transdetermined did not differ significantly from the clones which regenerated, either in terms of cell number or twin asymmetry (n = 5, Fig. 7B; compare purple and red points). This indicates that at the early time point, transdetermining twins have a doubling time that is not different from regenerating twins.

When 3/4L leg fragments were cultured longer, 13 days in vivo, clones that overlapped with Vg contained a median of 1930 cells, indicating about 11 doublings, compared to 10 doublings in transdetermined clones after 7 days of in vivo culture (n = 6, Fig. 7B, compare black and orange points). This indicates that after 7 days of in vivo culture, there is some, but little additional proliferation in transdetermined cells. While both groups were asymmetric in cell number per twin, the 13-day time point was not more asymmetric than the 7-day time point.

Our results indicate a distinct behavioral difference between regenerating (*ss-LacZ* expressing) vs. transdetermining (Vg expressing) clones. We asked if such a difference also occurs when anterior cells switch fate to regenerate the missing posterior compartment. We cultured 1/4 upper medial (1/4UM) prothoracic leg fragments, the complement to the 3/4L piece, in vivo. This fragment contains cells only of anterior identity and, as such, they lack engrailed (En) expression except in a few overlying peripodial cells (Gibson and Schubiger, 1999). However, after only 1–2 days of in vivo culture, the fragment upregulates En, and in doing so, the anterior cells regenerate the posterior compartment (Schubiger et al.,

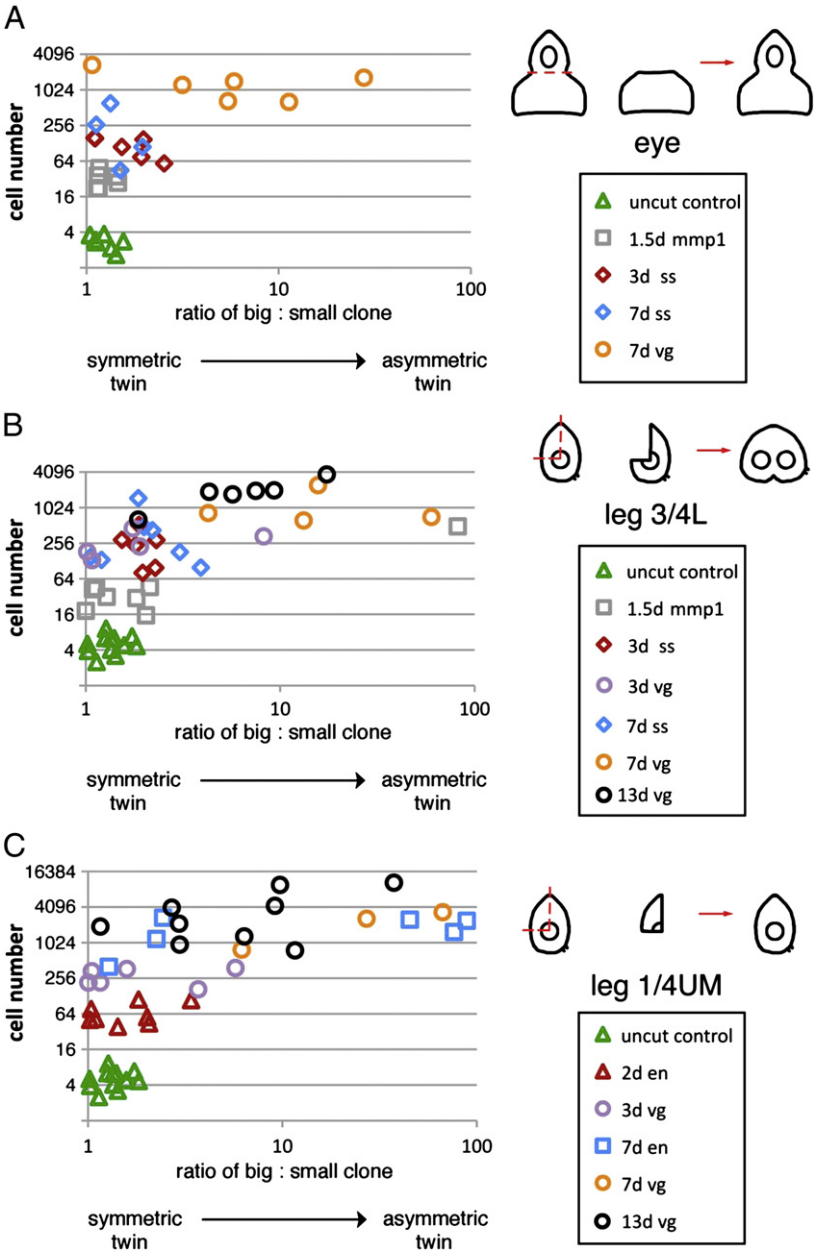

Fig. 7. As cells proliferate during regeneration, transdetermined sister clones are big and asymmetric. A summary of the twin spot experiments, including eye (A), leg 3/4L (B) and leg 1/4UM (C) fragments is plotted here. Each twin spot is represented by a single point. On the y-axis is cell number of the twin's larger clone. On the x-axis is cell-number ratio of each twin's big:small clone.

2010). We induced twin spots in 1/4UM fragments, cultured them in vivo for 2 days or 7 days, and stained the discs for En. After 2 days, the twins contained a median of 54 cells, and were relatively symmetric (n = 8, Fig. 7C, red points). After 7 days, the twins contained a median of 1970 cells (n = 6, Fig. 7C, blue points). Intriguingly, these six twin spots fell into two distinct classes on the scale of symmetry index. Additional staining of these fragments indicated that after 7 days of in vivo culture, fragments that expressed En often co-expressed Vg, and furthermore, in 50% of cases, MARCM clones that were En-positive were also Vg-positive (n = 8, data not shown). From this, we can presume that about half of the twins that expressed En were also undergoing leg to wing transdetermination, although in our assays with the twin spots, we could not address this directly. Nevertheless, in analyzing Vg-expressing twin spots in 1/4UM fragments that were cultured in vivo for 3 days, 7 days, or 13 days, we observed trends that supported our conclusion from the two other fragments, namely that transdetermining clones are large and asymmetric.

Together, our results indicate that transdetermined cells continue to proliferate after regeneration is complete. This is supported by evidence presented in Fig. 2D in which eye fragments, cultured in vivo for 7 days and labeled with a pulse of EdU, have a blastema that largely co-localizes with Vg and not *ss-LacZ* (Fig. 2D). To test this hypothesis further, we took advantage of the observation that, although fragments from the prothoracic leg disc can regenerate and transdetermine, an identical fragment from the mesothoracic leg disc regenerates but rarely transdetermines (Steiner et al., 1981). We cultured both types of disc fragments in vivo for 7 days, recovered them and labeled them with a pulse of EdU. We observed that the prothoracic leg disc fragment maintained a blastema in many cases (47%, n = 70), while the mesothoracic leg disc fragment did not (2%, n = 89). Again, this strengthens the hypothesis that transdetermination is strongly correlated with a continuation of cell proliferation.

Discussion

Elongation and separation of clones during regeneration

Our results provide novel evidence for clone elongation and possible cell displacement in imaginal disc regeneration. In other systems, cell migration has been implicated in both early and late phases of regeneration. For example, cell tracing in salamanders has shown that within 24 h of limb amputation, epithelial cells migrate to cover the wound, where they form the apical epidermal cap (AEC). It is thought that after migrating, these cells play a signaling role, rather than a major proliferative role, in limb regeneration (Hay and Fischman, 1961; reviewed by Whited and Tabin, 2009). In zebrafish, however, cell migration has been observed much later. Poleo et al. (2001) found that following fin amputation and proliferation, BrdU-labeled cells from the blastema cells migrate proximally and laterally, outside of the proliferation zone, prior to differentiating (Poleo et al., 2001). In our studies, we cannot distinguish between cell displacement during wound healing and later, during blastema growth. However two observations indicate that clone separation occurs later. First, Reinhardt and Bryant (1981), studying wound healing in imaginal discs with scanning electron microscopy, concluded that the cut epithelial edges come together without individual cell migration. Second, here we reported that the frequency of clone separation seems much higher at later time points, which suggests that the phenomenon occurs in the later phases of regeneration.

Recently, time-lapse microscopy of GFP-labeled mesenchymal cells in normal chick and mouse limb-bud development demonstrated that cells divide and migrate specifically along the proximo-distal axis. For proper orientation, Wnt5A, a Wg ortholog, and its downstream target c-Jun N-terminal kinase (JNK) are necessary (Gros et al., 2010). We speculate that this is a conserved mechanism that also orients cells during imaginal disc regeneration: both Wg and the JNK targets Puckered (Puc) and Matrix Metalloproteinase 1 and 2 (Mmp1 and 2) are upregulated at the wound after imaginal disc fragmentation, and are required for proper regeneration (Bosch et al., 2005; McClure et al., 2008). However, in all systems, because Mmps feed into so many pathways, it is unclear to what extent Mmp-mutant phenotypes, e.g., impaired regeneration, are simply due to a failure in wound healing, or in downstream events such as cell migration, proliferation, apoptosis or differentiation (reviewed by Bellayr et al., 2009). Previously we showed that loss of Mmp1 in leg disc regeneration dominantly disrupts normal blastema formation and enhances the area and frequency of transdetermination (McClure et al., 2008). Intriguingly, loss of Mmp1 also led to multiple spots of transdetermination at sites where it is normally not observed (McClure et al., 2008). We speculate that loss of Mmp1 leads to a dysregulation of normal cell movements. It would be interesting to test this directly in future studies.

Behavior of regenerating cells

Imaginal disc regeneration requires the proliferation of several highly plastic, seemingly immortal founder cells (Gehring, 1967; Gibson and Schubiger, 1999). This led us to wonder if imaginal discs contain a stem-cell-like population. Unfortunately there are no known markers to specifically identify these founder cells, as is the case in many stem cell systems. We used twin spot analysis to look for evidence of an asymmetrically dividing population. However, early in the regeneration process, we observed only symmetrically sized twin-spot clones. This does not exclude the possibility of stem cells. First, it is possible that clone induction was too early or too late and that we missed the critical time point for a stem cell division. Second, not all stem cell types divide asymmetrically. For example, in the developing *Drosophila* optic lobe, neural stem cells initially divide symmetrically to increase the precursor pool. Later some of the cells in this pool switch to asymmetrically dividing neuroblasts. It is thought that the switch between symmetrical and asymmetrical divisions, and the respective switch in proliferative potential, is a mechanism to regulate the relative numbers of differentiated neurons (Götz and Huttner, 2005). In the end, perhaps whether we consider imaginal disc regeneration progenitors to be “stem cells” is less important. Nevertheless we think our results do not favor a traditional stem-cell model for imaginal discs.

Do repeated switches in fate lead to immortality?

In regeneration, as well as during normal development, cells must stop dividing once the proper size of the organ has been attained. Failure to do so will lead to abnormalities or cancer. In normal *Drosophila* development, imaginal disc founder cells undergo about 10 doublings before differentiating into the adult cuticle (Bryant and Levinson, 1985; Madhavan and Schneiderman, 1977). Our results show that, upon fragmentation, cells regenerate with an additional 8 doublings. The twin clones proliferate in a symmetric manner and stop when regeneration is completed. Meanwhile transdetermining clones continue to proliferate to 10 doublings and more. Does a change in cell fate reset a cell's potential to divide?

The only proven immortal cell is the germ cell. However, we speculate that continual switches in fate, i.e. continual transdetermination, may induce a new type of immortal cell. Such speculation is inspired by results from Hadorn and co-workers, who, starting in the 1960s, passed disc fragments through series of in vivo culture periods in adults to test the concept of cell determination. Such proliferating “stem lines” were maintained for over five years, until they terminated the experiment with the conclusion that the cells were immortal. Along the way, they tracked disc identity by allowing test pieces to differentiate and analyzing the cuticular structures they formed. What was remarkable was that in the longest living cell lines, fragments continually switched fates. A genital disc fragment, for example, readily transdetermined to leg (1st order). If that disc fragment was cut and cell

division was stimulated again, then within the transdetermined leg, they found, for example, wing transdetermination (2nd order). Importantly, even after more than a decade of culture cells kept a normal haplotype with no obvious chromosomal aberrations and no contamination with host cells, and did not lose the capacity to differentiate (Hadorn, 1978).

Not all lines performed equally well. Strikingly, those that were healthiest and proliferated vigorously were the ones that had undergone the most transdetermination events, and where, for example, the disc identity continually switched from genital to leg, wing, notum, and antennal fates from one generation to the next. In contrast, the lines that died over time were the ones that, though continuing to regenerate their own disc type, settled into one fate (Hadorn, 1978).

In the experiments presented here, we have found the first direct evidence that switches in cell fate consistently correlate with an extension of cell proliferation, specifically at the site of transdetermination. The result is independent of disc type. We were intrigued to repeat Hadorn's long-term cultures with the twin spot generating system. We successfully maintained a leg disc stem line for 13 transfer generations over three months, however, we stopped the experiment after noticing that spontaneous clones were accumulating at each transfer generation (see Materials and Methods), likely due to the activation of *hs-flp* from the stress of transplantation. Since we could not know when clones were born, the results were impossible to interpret.

In the context of either short or long-term imaginal disc culture, we propose that immortality might be mediated by chromatin remodeling via Polycomb group (PcG) and trithorax group (trxG) genes. PcG and trxG genes are widely known to cause reversible homeotic transformations, including many transformations that phenocopy imaginal disc transdetermination (reviewed by McClure and Schubiger, 2007). Previously, our lab and others reported that PcG and trxG genes are mis-regulated during transdetermination and, when mutated, dominantly modify transdetermination frequencies (Klebes et al., 2005; Lee et al., 2005).

In addition to their role in modifying cell fate, PcG/trxG genes are also increasingly linked to a direct role in modifying proliferation in *Drosophila* and vertebrates (for a review see Martinez and Cavalli, 2006). Polycomb response elements (PREs) are predicted in the upstream regions of cell cycle genes such as Rbf, E2f, Dp, and Cyclin B (Oktaba et al., 2008), and loss or gain of PcG/trxG genes show strong under- or over-proliferation phenotypes. For example, loss of *Posterior sex combs (Psc)* along with its partner, *Suppressor of zeste 2 (Su(z)2)*, which we identified as a modifier of transdetermination (Klebes et al., 2005), is sufficient to upregulate Cyclin B and induce over-proliferation in wing disc clones (Beuchle et al., 2001). Oktaba et al. (2008) also reported that such mutant clones contain larger cells with a dramatic shift from G1 into S and G2/M, intriguingly, the same cell cycle shift that we observed in transdetermining cells (Sustar and Schubiger, 2005).

In summary, we report here that transdetermined cells have an extended proliferative life, and we speculate that such fate changes are sufficient to reset the proliferation potential. As clonal analysis and other cell-marking tools become more common in other systems, it will be interesting to see if this is a common mechanism by which cells can attain unlimited proliferation.

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